



Review

# The use of thiophilic chromatography for antibody purification: a review

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## 1. Introduction

The association between thiophilic sorbents and immunoglobulin separation started with the demonstration by Porath et al. [1] that it was possible to fractionate plasma proteins by chromatography on an agarose-based sorbent resulting from the reaction of divinylsulfone and 2-mercaptoethanol. Since then, this mode of protein separation was mostly applied to antibody purification because the sorbent displays a certain specificity for this class of proteins [2,3]. This discovery was an important step in the development of separation methods for antibodies involving, as chromatographic ligands, a large variety of structures that contain sulfur and nitrogen atoms having a relatively high level of selectivity. Sorbents based on this technology were and still are serious challengers to bioaffinity chromatography, involving *Staphylococcus aureus* Protein A [4–6].

Structurally, the most common sorbents for thiophilic chromatography resulting from the above-mentioned reaction are called “T-gel”; they carry linear ligands with two sulfur atoms. These derivatives have shown fairly good selective binding for immunoglobulins in the presence of high concentration of structure-forming salts (also called lyotropic salts), such as ammonium sulfate or sodium sulfate. In its behavior, thiophilic-adsorption chromatography resembles hydrophobic-interaction chromatography: adsorption is promoted by highly concentrated salts, and elution occurs when the salt concentration is lowered. However, hydrophobic associations and ionic interactions do not occur with thiophilic sorbents since thio-ethylsulfone structures do not possess pronounced hydrophobicity, on one hand, and do not contain ionic charges, on the other

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hand. The type of salt also influences the strength of the interaction. Sulfates and phosphates are mostly used in thiophilic-mediated chromatography, while the presence of sodium chloride even at high concentrations, does not promote adsorption of proteins.

Recent studies [7] of a series of different sorbents—all of them representative of salt-promoted adsorption—showed that thiophilic solid phases, based on electron donor acceptor properties, are distinct from hydrophobic sorbents. This category of chromatographic material displays high levels of selectivity for immunoglobulins, contrary to hydrophobic solid phases with aliphatic ligands that adsorb hydrophobic proteins. It should be noted that hydrophobic-interaction sorbents, based on aromatic ligands, seem to behave towards immunoglobulins in a manner somewhat intermediate between genuine T-gel supports and alkyl ligands supports [8]. Interest in the interaction between the original thiophilic ligand structures and antibodies led to the development of a number of chemical derivatives intended to improve the specificity for antibodies and to increase binding capacity as well as stability of the sorbents. These improvements are a necessary response to the increase demand for antibody purification. Today, antibodies are very important for a large variety of applications. They are and will continue to be at the forefront of therapeutic as well as diagnostic applications. Whether from natural sources or from recombinant cells, they must be extracted and purified. The degree of purity depends on the application, but the trend is toward higher purity while preserving biological activity at lower and lower cost. This situation is very conducive to the discovery of more effective production and purification technologies that meet regulatory requirements. Downstream processing, with its important chromatographic separation component, is critical. Therefore, new separation methods appear and current technologies are revised in order to make them more specific and economically viable.

Thiophilic-adsorption chromatography has been described for the purification of a large number of antibodies from different species and also for difficult separations such as the purification of murine monoclonal antibodies from hybridoma cell culture containing fetal bovine serum [9]. Immunoglobulins have been selectively separated from bovine colostrum and milk whey [10–12], where no specificity for different classes was found. Variations in the structure of the original thiophilic spacers by, for instance, the introduction of amine groups, resulted in new features, such as greater stability in strong alkaline media and adsorption of antibodies with a reduced level of ionic strength [13]. However, these changes did not improve the selectivity for antibody classes. A similar lack of class-specificity discrimination was also observed for human immunoglobulins from blood [14] and from hybridoma cell culture supernatants, where the binding capacity of the resin and the purity of the antibodies did not differ significantly from one antibody to another, except for IgG2a, which seemed less pure than other classes.

Thiophilic sorbents in the presence of specific salts have been used to separate antibodies from crude ascites fluids with good results in a one-step procedure [15].

Cell culture supernatants have also been used in a variety of cases for the separation of antibodies by thiophilic chromatography. Birkenmeier and Kopperschlager [16] have reported the separation of IgG1 monoclonals against horseradish peroxidase in conjunction with a two-phase extraction procedure. This separation method compared advantageously with other chromatographic techniques, such as ion exchange, Protein A affinity

chromatography, and hydrophobic-interaction chromatography [17]. Purification of several preparations of monoclonal IgG2b, obtained by culturing cells in serum-containing media or protein-free conditions, was tried with thiophilic gels, producing final purity of at least 97% [18].

Recently, the separation of IgY from egg yolk by thiophilic chromatography has been described. The recovery in the presence of ammonium sulfate was close to 100%, and the binding capacity was in the range of 25 mg of antibodies/ml of resin [19]. Still in the domain of antibodies, thiophilic chromatography has been used for the purification of fragments obtained by the enzymatic breakdown of immunoglobulins [20,21]. Fab fragments, expressed by recombinant *E. coli*, were also separated by thiophilic chromatography. Adsorption was achieved as usual at high concentrations of ammonium sulfate and desorption by lowering the ionic strength [22].

All of these examples are witnesses to the great potential of thiophilic chromatography in the purification of antibodies. Today, thiophilic chromatography techniques in their latest versions are being developed at an industrial scale; they involve mostly ligands structured in such a way that they are composed of heterocycles, with nitrogen atoms or sulfur or both, having a predominant ability to separate antibodies from various crude feed stocks under the most specific conditions. The development of this separation technique occurred at a crucial time when the needs of antibody purification became urgent as a result of their development for medical and diagnostic applications.

The purpose of this review is to update of an attractive, relatively old method of antibody separation, known under the term of thiophilic chromatography, which is characterized by an extensive evolution over the last two decades. The last part of this review covers recent developments of the original technology towards a greater selectivity and binding capacity for antibodies under physiological conditions.

## 2. History and basis of thiophilic chromatography

The importance of sulfur atoms for ligand adsorption chromatography was recognized ca. 40 years ago [23,24]. Aromatic adsorption was one of the phenomena investigated at that time, and it was found that when aromatic adsorption was mediated by the immobilization of 2,4-dinitrophenyl groups, a synergistic adsorption effect was evidenced when the ligate contained a sulfur atom instead of oxygen atom close to the aromatic ring. This was one of the first indications that the sulfur atom, as part of a ligand, has an effect on the adsorption of a solute. The strength of this interaction appeared dependent of electrophilic and nucleophilic ring substituents; in fact, adsorption by electron donor–acceptor complexes was indicated to be at the basis of the interaction.

In the seventies with the development of a number of activation schemes for the immobilization of ligands for affinity chromatography, divinylsulfone was used as activating reagent [25]. It was found that, when the vinylsulfone-activated groups were blocked with 2-mercaptoethanol, the resin became a good adsorbent for proteins [1,2,26]. Both functional groups, sulfone and thioether, present in the ligand structure,

appeared to act in a cooperative manner to exert strong protein affinity. This adsorptive strength was enhanced by the addition of sulfate-containing buffers. One may argue that this is similar to hydrophobic-interaction chromatography, where the presence of sulfate salts is an important parameter for protein adsorption. However, these compounds, resembling those illustrated in Fig. 1, are hydrophilic structures. When even more hydrophilic endcapping groups, such as 3-mercaptopglycerol are used instead of 2-mercaptoethanol, the resulting structure (cf. Fig. 1) is even more hydrophilic but shows similar adsorption characteristics as the previously mentioned structure in the presence of sulfate buffers. Based on these findings, this kind of adsorption was characterized as thiophilic chromatography.

Elution patterns of human serum as complex protein mixture, chromatographed on thiophilic agarose gels, also called T-gel, evidenced the good separating properties of this material [27]. Adsorption was performed in highly concentrated ammonium sulfate and followed by elution with a lower concentration of ammonium sulfate in the buffer, showing selective protein desorption. Good separation was also observed, e.g., for immunoglobulins G from  $\alpha$ 1-antitrypsin and from  $\alpha$ 2-macroglobulin. Other protein components were also separated. To compare separation capabilities with T-gel, the elution profile was compared to the one obtained under the same conditions but with a hexyl-agarose material and very different results were obtained. Albumin was eluted before IgG and before  $\alpha$ 1-antitrypsin. Although adsorption elution conditions for thiophilic-interaction chromatography resemble those in hydrophobic-interaction chromatography, several differences indicate that they are two distinct methods of protein separations (see Table 1). It is known that ligands in hydrophobic-interaction chromatography are hydrophobic due to the presence of aliphatic chains and/or aromatic non-ionic rings. In contrast, classical thiophilic ligands are hydrophilic [26]. Contrary to thiophilic interaction [1,28], hydrophobic interaction increases with temperature during the adsorption phase. Hydrophobic interaction is not promoted only by lyotropic salts, but also by sodium chloride, while the latter promotes desorption from thiophilic adsorbents. It should also be noted that albumin, which is strongly adsorbed on hydrophobic ligands [29], is weakly adsorbed on thiophilic solid phases [30]. When plasma proteins were

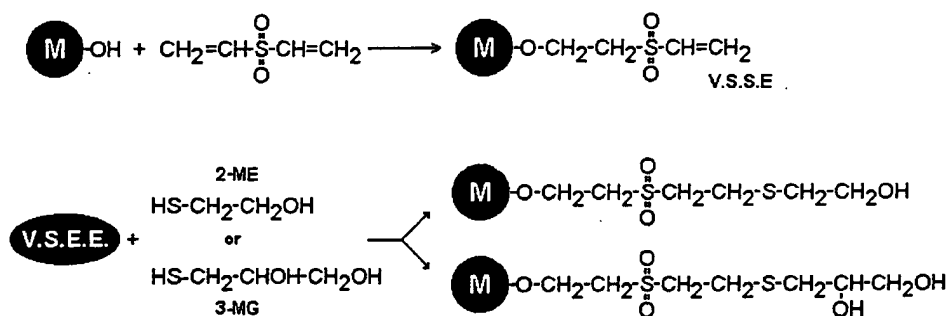


Fig. 1. Preparation of thiophilic "T-gel": chemical reaction schemes and structure. M = matrix (e.g., agarose); V.S.E. = vinyl-sulfonyl-ethyl ether; 2-ME = 2-mercapto-ethanol; 3-MG = 3-mercaptopglycerol.

Table 1  
Differences between thiophilic-interaction chromatography (TIC) and hydrophobic-interaction chromatography (HIC)

	TIC	HIC
Ligand character	Mostly hydrophilic	Hydrophobic
Interaction increases with temperature	No	Yes
Lyotropic salts promotes interaction with proteins	Yes	Yes
Sodium chloride promotes interaction with proteins	No	Yes
Adsorption of albumin	Weak	Strong
Adsorption of immunoglobulins G	Yes	Weak for aliphatic ligands

loaded onto a thiophilic sorbent and hydrophobic sorbents in 1 M ammonium sulfate solution, differences appear in the separations [14] (see Fig. 2). With thiophilic material, no differences in subclasses were found, while with octyl-agarose resin, in spite of a poorly adsorbed fraction, an enrichment in IgG2 was found. In the analysis of non-adsorbed fractions obtained at different concentrations of ammonium sulfate, it was additionally observed that interaction properties of thiophilic sorbents are different from those of hydrophobic sorbents, at least for the adsorption of immunoglobulins G. However, even considering that molecular interactions at the basis of protein separation on thiophilic sorbents are different from those on hydrophobic sorbents, they showed some similarities in the elution of several proteins (Fig. 2). From these preliminary data, it was clearly evident that thiophilic chromatography would play an important role in protein separation, especially if they are to be processed at nearly neutral pH [27].

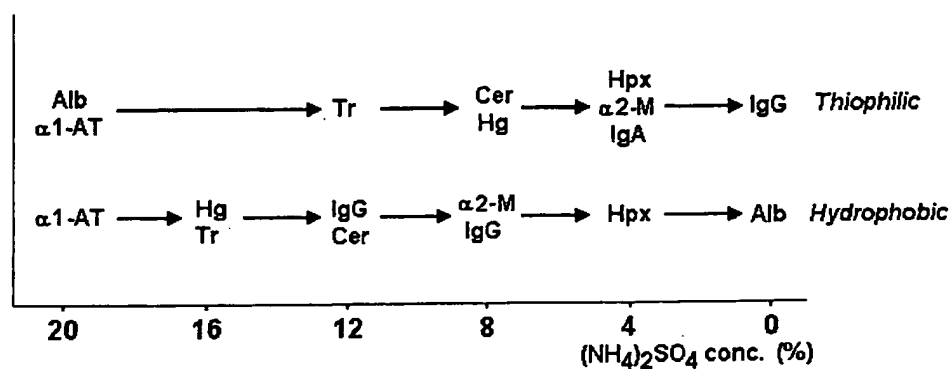


Fig. 2. Order of elution of serum proteins from a thiophilic sorbent compared to a hydrophobic sorbent by step gradient elution with ammonium sulfate. Adapted from Ref. [27]. Residual proteins still adsorbed on the column are eluted with ethylene glycol up to 40%, according to the authors. Alb = albumin;  $\alpha$ 1-AT =  $\alpha$ 1-antitrypsin; Tr = transferrin; Cer = ceruloplasmin; Hg = haptoglobin; Hpx = hemopexin;  $\alpha$ 2-M =  $\alpha$ 2-macroglobulin; IgA = immunoglobulin A; IgG = immunoglobulin G.

Affinity constants of pure immunoglobulins on thiophilic sorbents increase with the concentration of lyotropic salts [2,28] and may approach values of  $10^5$ – $10^7$  M<sup>-1</sup>. However, at high salt concentration (e.g., ammonium sulfate), immunoglobulins precipitate. Therefore, a compromise must be found between precipitation and adsorption selectivity on thiophilic sorbents [2,30].

In a comparative experiment [27], it was demonstrated that thioether and sulfone groups have a synergistic effect in their ability to adsorb proteins in a manner different from hydrophobic chromatography. Chromatograms of several proteins on T-gel and on a hexylsulfido-agarose are, in fact, different, even though both contain sulfur atoms (see schematic representation of elution patterns in Fig. 2). However, other proteins are similarly located on both chromatograms, indicating that the effect of hydrophobicity is not completely absent from thiophilic T-gel sorbents. Protein adsorption correlates with the electron-donor properties of some amino acids in proteins. Affinity loci in protein structures, where the interaction with thiophilic sorbents presumably happens should consist of peptide segments containing tryptophan, phenylalanine and tyrosine, which are considered to be electron donors with strength in the order just given [31]. It was demonstrated that, when a single alanine was substituted by phenylalanine, the retention is significantly enhanced, indicating that an electron-transfer phenomenon is involved [22].

There may be situations where the interaction is more complex as a result of a multitude of interacting points due to the vicinity of thiophilic ligands on the solid matrix and to the presence of not only donor, but also electron-acceptor sites on the protein itself. As will be discussed later, the presence of other chemical groups, located proximal to the thioether bond, e.g., heterocycles, enhances the adsorption of proteins and especially the specificity for immunoglobulins. As an example, if the group vicinal to the thioether bond is a pyridine ring, IgG is adsorbed much strongly than when a phenyl group is present [32].

General reviews on adsorption-desorption phenomena dependent on the electron donor-acceptor principle and involving sulfur atoms describe the interaction mechanisms in some details [27,32,33].

### 3. Antibody purification requirements

Antibodies as tools for research and for diagnostic and therapeutic applications have proliferated since the discovery of monoclonal antibodies by Köhler and Milstein [34]. This evolution accelerated more recently when new possibilities in human therapy opened up.

In the last few years, monoclonal antibodies and recombinant antibody constructs have become the largest class of proteins that are currently in clinical trials and have received FDA approval as therapeutics and diagnostics. This is the result of a large research investment, in particular, the introduction of man-made molecules and the improved definition of targets. As a consequence, significant progress in the understanding of antibody function, of host-defense mechanisms and of the role of antibodies in

cancer and substantial improvements in production and purification technology have been achieved. Development of protein-free culture media, continuous production of animal cells in perfusion culture, genetically engineered “humanized” antibodies [35], development of single-chain antibodies, phage display [36,37] and cell-surface display libraries, have been important steps in this process.

Design of antibodies according to special needs of therapy, diagnosis, and purification technology is now possible. Specific properties for favorable *in vivo* behavior, such as defined pharmacokinetics and tumor targeting [38], are simply achieved by combining various fragments with desired properties. These highlights are only a few examples of ongoing progress. Antibodies are easily expressed by hybridoma cells or by other recombinant technologies. Expression systems, such as bacteria [39], yeast [40], insect cells [41], and mammalian cells [42], are currently used for the expression of antibodies and fragments thereof. However, due to renaturation problems, glycosylation, and expression levels, mammalian cells were and are still the technology of choice for the expression of monoclonal antibodies. More recently technologies have been developed for the expression of antibodies in transgenic animals [43] and transgenic plants [44] (cf. Table 2).

Complementary to expression systems and production strategies are purification protocols designed to obtain very pure antibodies in a simple manner and at low cost. Purity, safety, potency, and cost effectiveness are, in fact, the main points to consider when designing the expression method and, more importantly, when defining purification processes.

The purification of antibodies has a history that commenced with the separation of proteins several decades ago. A large number of methodologies have been suggested, involving precipitation methods, electrophoretic separations, membrane filtration and liquid chromatography. The latter represents probably the most popular technique because of the ease of implementation, of the capability to manipulate the selectivity, and because of the level of purity that can be achieved.

Table 2  
Most important expression systems for antibodies and their fragments

	Expression level (mg/ml)	Starting material for purification	Glycosylation
Mouse, rat ascites	1–10	Ascites	Yes
Hybridoma culture	0.05–0.5	Extracellular, supernatant	Yes
Recombinant eukaryotic cells culture	0.05–0.8	Extracellular, supernatant	Yes
Yeasts	0.02–0.2	Extracellular, supernatant	Yes
Bacteria	0.02–0.1	According to the expression system	No
Transgenic animals	1–10	Milk	Yes
Transgenic plants	0.1–0.3	Seeds, leafs, apoplastic space	No
Eggs	2–8	Yolk or white	Yes

Specific liquid-chromatographic methods and resins have been developed especially for this purpose based on a variety of adsorption/desorption mechanisms. However, although antibodies are very diverse in biological properties, they have several properties in common that are frequently exploited for their capture from the initial feed stock. It is known that several physico-chemical and structural similarities are shared between antibody molecules, and that is why they are considered to be a homogeneous group of proteins.

Valid for any protein separation problem, another point to consider, which is frequently a key for success, is the knowledge about the nature and concentration of the impurities. Thus, the initial composition of feed stock is of importance when designing antibody separation processes.

As the cost of antibody production becomes critical, methods less expensive than affinity chromatography, such as Protein A chromatography, are being considered. In this context, thiophilic chromatography and related techniques with specific features look more and more attractive.

While whole antibodies vary greatly in specificity, they have common structures with well-established, highly conserved regions that can be used for their separation from complex feed stocks. The ideal approach would be to start with crude material and load it directly onto a chromatographic column without changes in pH, ionic strength and concentration. This approach is already applied most often with Protein A sorbents. However, performance in terms of longevity, stability, price, leakage of ligand, binding capacity, and cleanability with sodium hydroxide is not ideal. For this, specific ligand design or selection is required. Selectivity for the highest purity in such a model would mean not only manipulation of the adsorption step, but also the desorption, e.g., with competing agents.

Most recent developments in thiophilic chromatography seem concerned with the right response to the above-mentioned requirements.

#### **4. From divinylsulfone activation to multi-S linear thiophilic ligands**

Among chemical activation methods for ligand immobilization on solid phases, divinylsulfone was described more than two decades ago as an efficient way to obtain reactive sites [25,45,46]. Vinylsulfone groups are introduced in polysaccharide matrices, such as agarose, by a reaction mechanism similar to that of bisepoxyranes [47]. The vinyl derivative thus obtained is more reactive than the corresponding epoxy-containing matrix. This activation reaction is at the basis of a large number of affinity chromatography resins, where the ligand is attached after divinylsulfone activation. Activated polysaccharides can react either with primary-amino-containing ligands or with mercapto ligands. In both cases, the ligands are covalently attached and are stable in a wide pH range. However, a very high pH may damage the linkage with consequent partial release of the ligand [48]. One of the most popular derivatives of agarose, activated with divinylsulfone, is a derivative called thiophilic sorbent or T-gel. The material contains two sulfur atoms and is obtained by reacting 2-mercaptoethanol on divinylsulfone-activated agarose beads (Fig. 1).



These derivatives showed specific binding for IgG in the presence of high concentrations of lyotropic salts [26]. Ligand densities between 15 and 1000 mol g<sup>-1</sup> gel (dry mass) were obtained [30]. Maximal binding capacities for antibodies differed when pure IgG (0.65 g/g of dry gel) was compared with serum (0.1–0.15 g/g of gel). Apparent, affinity constants for pure immunoglobulins in the presence of 750 mM ammonium sulfate in a buffer of pH 7.5 were between 10<sup>5</sup> and 10<sup>7</sup> (Table 3). However, it was emphasized that antibodies precipitate in the presence of high concentrations of lyotropic salts and also that the binding capacity and affinity constant increase as the concentration of lyotropic salts increases, while the specificity decreases. Simple thiophilic sorbents, structured as T-gel, have been used for the separation of a large variety of antibodies from different species and feed stocks, alone or in combination with other separation techniques (cf. Introduction).

Nopper et al. [49] have indicated that the structure of T-gel sorbents could be modified to increase their specificity and binding capacity. Linear ligand structures with from three to six sulfur atoms were described [49,50]. Their structures are illustrated in

Table 3  
Associations constants of immunoglobulins G on several thiophilic sorbents in different buffer conditions

Structure of thiophilic ligands	Buffer conditions	Apparent association constant (M <sup>-1</sup> )	Reference
DVS-activated agarose	0.8 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.9 × 10 <sup>7</sup>	[52]
T-gel	PBS + 0.5 M K <sub>2</sub> SO <sub>4</sub> , pH 7	2.5 × 10 <sup>6</sup>	[13]
T-gel	PBS, pH 7	9 × 10 <sup>3</sup>	[13]
T-gel	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	1.5 × 10 <sup>6</sup>	[50]
Aminoethyl-sulfonyl	PBS + 0.5 M K <sub>2</sub> SO <sub>4</sub> , pH 7	3.1 × 10 <sup>7</sup>	[13]
Aminoethyl-sulfonyl	PBS, pH 7	2.3 × 10 <sup>5</sup>	[13]
3S (see Fig. 3)	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	8.7 × 10 <sup>7</sup>	[50]
4S (see Fig. 3)	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	2.4 × 10 <sup>7</sup>	[50]
5S (see Fig. 3)	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	5.2 × 10 <sup>9</sup>	[50]
6S (see Fig. 3)	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	8.6 × 10 <sup>11</sup>	[50]
4S + 2O (see Fig. 3)	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	5.5 × 10 <sup>6</sup>	[50]
4S + 2N (see Fig. 3)	10 mM HEPES + 275 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	3.1 × 10 <sup>6</sup>	[50]
Pyridyl-thio-hydroxypropyl	10 mM HEPES + 400 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	7.0 × 10 <sup>7</sup>	[50]
Pyrimidyl-thio-hydroxypropyl	10 mM HEPES + 400 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	5.8 × 10 <sup>7</sup>	[50]
Thiazolyl-thio-hydroxypropyl	10 mM HEPES + 400 mM Na <sub>2</sub> SO <sub>4</sub> , pH 7.4	4.1 × 10 <sup>7</sup>	[50]
Mercapto-ethyl-pyridyl	PBS, pH 7	2.9 × 10 <sup>7</sup>	[70]

DVS = divinylsulfone.

Fig. 3. Affinity constants were determined (Table 3), and all derivatives were evaluated for their ability to separate IgG. All of these structures required relatively large amounts of sodium sulfate to promote IgG adsorption; the pH for adsorption was always between 5 and 9, while elution occurred at pH 3. The binding capacity obtained with this material was between 18 and 28 mg of IgG/ml of sorbent, depending on the nature of the solid matrix. However, it has been found that, with cell culture supernatants containing phenol red as pH indicator, the binding capacity decreased to a few mg of IgG/ml of resin as a result of molecular competition. This study demonstrated that the more sulfur atoms are part of the ligand, the higher is the affinity and the binding capacity for antibodies. However, even if the number of sulfur atoms is relatively high (e.g., 4), their connection with oxygen or nitrogen atoms decreased the affinity constants significantly (Table 3). Despite greater specificity for antibodies, sorbents containing long chains with several sulfur groups have remained a good theoretical concept without practical preparative applications.

In a thorough study of the conditions used to prepare T-gels, Scolbe and Scopes [51] have demonstrated that end-capping a divinylsulfone-activated agarose with 2-mercapto-ethanol is not essential for obtaining a good sorbent for antibody interaction. In fact, if the divinylsulfone-activated agarose is treated in strongly alkaline media to hydrolyze the vinyl group and thus substitute an S group by an O group, the final sorbent retains its ability to adsorb IgG. If a thiophilic sorbent is treated with a strong reducing agent, such as sodium borohydride, its ability to adsorb IgG decreases significantly. Additionally, it

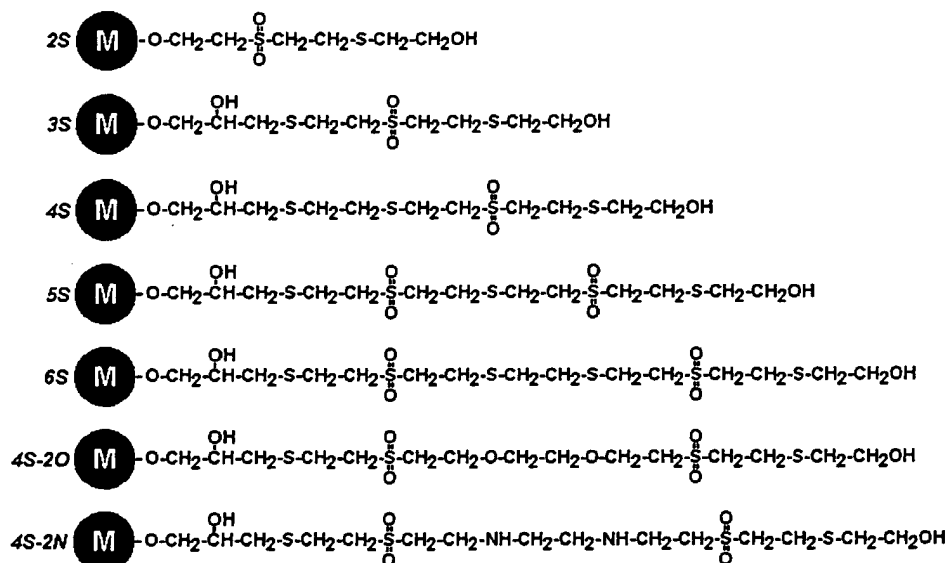


Fig. 3. Structural representations of thiophilic ligands attached on a matrix (M) solid phase. Ligands contain an increased number of sulfur atoms (from 2 to 6). In structures 4S–2O and 4S–2N, two atoms of sulfur at the center of the chain are replaced by either two atoms of oxygen or two atoms of nitrogen, respectively. End-capping was always performed with 2-mercapto-ethanol (see Fig. 1). Adapted from Ref. [54].

was found that extensive activation with divinylsulfone resulted in a sorbent with larger binding capacity.

Noel et al. [52] have reported that a divinylsulfone-activated matrix *per se* purifies immunoglobulins according to the same mechanism as that described for T-gels. This work suggested that divinylsulfone can form dimers, connected with an ether linkage, indicating that divinylsulfone-activated matrices fall into the class of ligands described as thiophilic.

Regular thiophilic sorbents, prepared by first allowing divinylsulfone on agarose to react and then end-capping with 2-mercaptoethanol, also produce a considerable amount of cross-linking [25] and can be easily hydrolyzed under strongly alkaline conditions. Both facts limit the binding capacity and the possibility to clean the sorbents with sodium hydroxide.

Further investigations were extended to thiophilic sorbents involving spacer arms with amine groups [13]. The binding capacity of these derivatives was dependent on ligand density. According to the authors, thiophilic sorbents made with amino spacers showed a similar binding capacity as T-gel calculated on the basis of dry material. The affinity constant for IgG is higher than with T-gels. This feature allows adsorbing antibodies from more dilute feed stocks. The divinylsulfone activation reaction has been used as a linker for the immobilization of several aromatic and heterocyclic compounds on chromatographic sorbents for immunoglobulin separation. These developments are discussed in Sections 5 and 6.

## 5. Aromatic thiophilic sorbents

Ligands consisting of aromatic structures immobilized with a spacer arm that contains sulfur atoms have been evaluated for the purification of antibodies. From the literature, two approaches can be identified: one is the immobilization of aromatic rings, such as aminobenzoic acid and methoxyphenol after activation of the solid matrix by divinylsulfone [53]; the other one is the immobilization of aromatic mercaptans on epoxy-activated resins [33]. Structurally, these resins are very different, as shown in Fig. 4. These two types of resins are described as being able to adsorb proteins in a manner different from the case of regular hydrophobic interactive aromatic resins. However, their specificity for immunoglobulins seemed relatively modest for both of them. For instance, 3-(1-phenylsulfido)-2-hydroxypropyl-agarose adsorbed albumin in addition to immunoglobulins and several other plasma proteins to a smaller extent. Adsorption was salt-dependent, being possible only in the presence of 0.5 M potassium sulfate in Tris buffer (pH 7.5). Although 4-aminobenzoic acid and 4-methoxyphenol, attached to agarose via divinylsulfone activation, were able to adsorb gammaglobulins, several other proteins were also adsorbed in the presence of 0.75 M ammonium sulfate. Clearly, these sorbents are much less selective for immunoglobulins than are regular T-gels. Porath and Oscarsson [33] suggested that such ligands are intermediate between thiophilic and hydrophobic sorbents. In this respect, it should be recalled that aromatic hydrophobic-interaction chromatographic supports without sulfur in the spacer or in the phenyl ring adsorb monoclonal antibodies in the presence of highly structured salts at 1 M

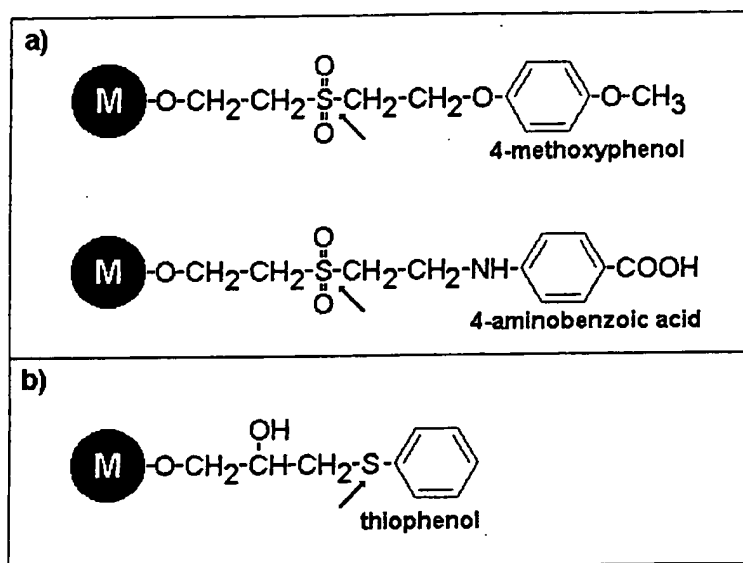


Fig. 4. Representation of aromatic thiophilic sorbents resulting from the coupling of aromatic ligands on divinylsulfone-activated matrix (a) or from the coupling of aromatic mercaptans on epoxy-activated matrix (b). In the first case, the sulfur atom is introduced by the activating reagent and is positioned on the spacer arm; in the second case, sulfur is proximal to the aromatic ring [33,53]. Arrows indicate sulfur atom positioning. M = matrix.

concentration and at pH 7. A purification of monoclonal IgG2a on such a support has recently been reported [8].

## 6. Heterocyclic thiophilic chromatography

The use of heterocyclic compounds for the synthesis of thiophilic ligands was introduced by Porath and Oscarsson [32,33]. It was demonstrated that aromatic or heterocyclic compounds, attached to a solid matrix via a thioether bond, were able to adsorb some defined proteins from plasma, such as antibodies,  $\alpha$ 2-macroglobulin, but not albumin. These proteins are the same as those adsorbed on the thiophilic resin T-gels. This suggested that the presence of sulfur atom is essential for introducing selectivity for given groups of proteins. However, it has been observed that several other proteins could also be adsorbed, indicating that the presence of the aromatic or heterocyclic group was also cooperating in the adsorption. This specific influence was induced by the  $\pi$ -electron system of the pyridine residue. Adsorption of immunoglobulins is effective in the presence of lyotropic salts, as discussed above in Sections 2 and 4.

To demonstrate the influence of different pieces that constitute the ligand construct, variants were made. In addition to the ligand composed of a pyridine ring attached to the matrix via a thioether linkage, three other structures were made, combining pyridine and

phenyl rings anchored to the matrix with a sulfur or oxygen atom [33]. Structures involving a pyridine ring and a sulfur atom adsorbed mostly immunoglobulins and  $\alpha$ 2-macroglobulin when whole serum was loaded on the column. When a phenyl group replaced pyridine, albumin was adsorbed in addition to immunoglobulins. Adsorption was in both cases salt-promoted. In the two cases where the sulfur atom was replaced by an oxygen atom, no selective adsorption of protein was observed. These findings clearly demonstrated that a sulfur atom is necessary for salt-promoted adsorption and that pyridine cooperates in the specificity toward antibodies.

Oscarsson and Porath [32] have investigated a number of sorbents based on pyridine and alkyl-thioether agarose with the aim to identify the greatest specificity for the adsorption of immunoglobulins [32]. Among the structures reported, they classified thio-pyridine as more efficient than thio-phenol for the adsorption of antibodies and non-adsorption of albumin. The high level of interaction of albumin with thio-phenol compared to thio-pyridine seemed to reflect the higher level of hydrophobic association. A series of heterocyclic thiophilic sorbents is shown in Fig. 5.

The ability of these thiophilic structures to adsorb proteins implies the presence of a sulfur atom. However, this is effective only if it is combined with a fully aromatic group or with a pyridine ring. The attachment of a pyridine ring to the sulfur atom also influences the ability to adsorb proteins and the selectivity of the adsorption. Thus, 4-thiopyridine adsorbs a larger amount of proteins than 2-thiopyridine and is more

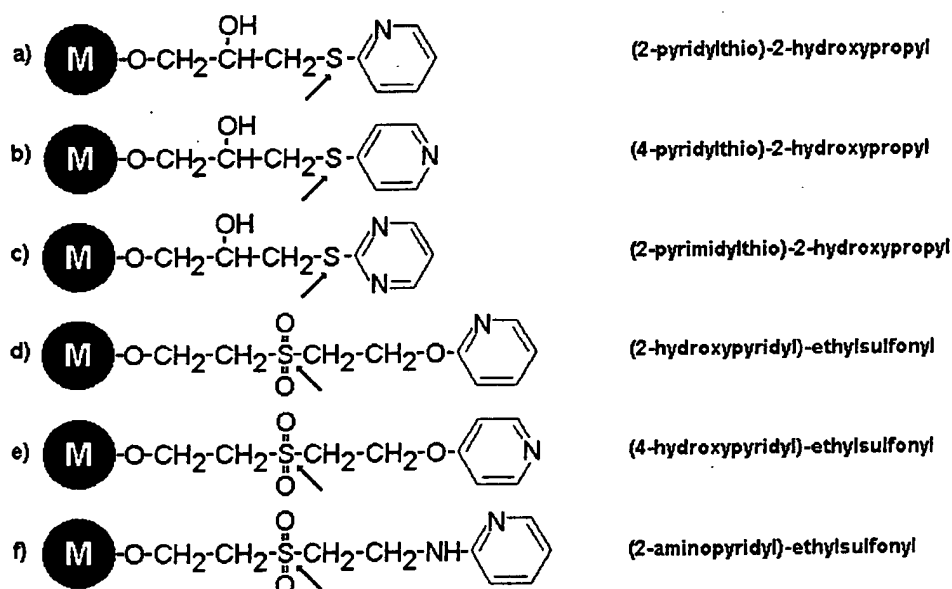


Fig. 5. Representation of heterocyclic thiophilic resins made according to activation methods shown in Fig. 4. The sulfur atom is part of the spacer and is located either proximal to the heterocycle ("a", "b" and "c") or in the middle of the spacer ("d", "e" and "f"), as indicated by the arrows. Sorbents "a" to "c" result from epoxy activation; sorbents "d" to "f" result from divinylsulfone activation.

selective for immunoglobulins, while the latter indiscriminately adsorbs  $\alpha$ 2-macroglobulin and immunoglobulins.

Other thiophilic matrices composed of hetero-aromatic ligands attached via divinylsulfone activation have been described by Knudsen et al. [53]. These authors described heterocyclic ligands for the purification of human and mouse immunoglobulins. Agarose beads were first derivatized with divinylsulfone and then various aromatic structures were immobilized. Among them, 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine, 2-aminopyridine, and imidazole were tested for the isolation of IgG from human serum. These ligands exhibited a remarkable binding capacity, but still required the presence of lyotropic salts. Adsorption of immunoglobulins from serum was performed with 1.2 M ammonium sulfate in 50 mM acetate buffer (pH 5.2), followed by a washing with a solution of similar composition but with an ammonium sulfate concentration decreased to 0.3 M. Elution of IgG was performed using 50 mM Tris buffer (pH 9). In all cases, the IgG collected was still contaminated with other serum proteins. However, purity could be improved by an additional washing step with a buffer, containing polyethylene glycol. Monoclonal IgG1 was purified from a cell culture supernatant, using 2-hydroxypyridine-agarose, with a yield of 71% and a purity of 90%. Binding capacity for IgG under these conditions was in the range of 30–60 mg/ml of resin. Antibody binding was influenced by the structure of the aromatic moiety of the ligand, while thiophilicity was brought about by the divinylsulfone activation. Hydroxypyridine seemed like the preferred ligand, able not only to adsorb immunoglobulins, but also to discriminate best between albumin and IgG. Adsorption required a significant amount of lyotropic salts, as shown for all other thiophilic ligands.

Mercapto-pyridine ligands attached to agarose beads were used as thiophilic adsorbents for antibodies in RIA and ELISA procedures [54]. As observed for the linear thiophilic ligands, all these sorbents required relatively high concentrations of potassium sulfate. This salt also promoted the adsorption of immune complexes formed in solutions, suggesting that adsorption sites are present in the Fc fragment of immunoglobulin molecules. Human, mouse, goat, and rabbit IgG were equally adsorbed on these thiophilic resins.

Divinylsulfone-activated agarose was also end-capped by several heterocyclic compounds, and the performance of the resulting sorbents was compared to regular T-gel [52]. Tryptophan, hydroxypyridine, hydroxyquinoline, and pyridine were coupled to the activated matrix; all these derivatives evidenced a clear ability to separate immunoglobulins from cheese whey. However, the purity of IgG varied according to the end-capping compound.

In 1995, Schwarz et al. [55] described novel heterocyclic structures able to adsorb immunoglobulins selectively. These structures contained sulfur and nitrogen; for the first time, the sulfur atom was moved from the spacer arm position to the heterocycle ring itself. Reported ligands were: 2-mercapto-pyridine, 2-mercapto-pyrimidine, and mercapto-thiazoline. These structures were chemically immobilized on silica and agarose beads using epoxy-activated matrices (see Fig. 6). Binding capacities of silica-based materials were about 25 mg of IgG/ml of resin, while it was about 18 mg/ml for agarose beads. IgG was separated from ascites fluid using these resins; adsorption was performed in the presence of 350 mM sodium sulfate at pH 7.4, and elution was

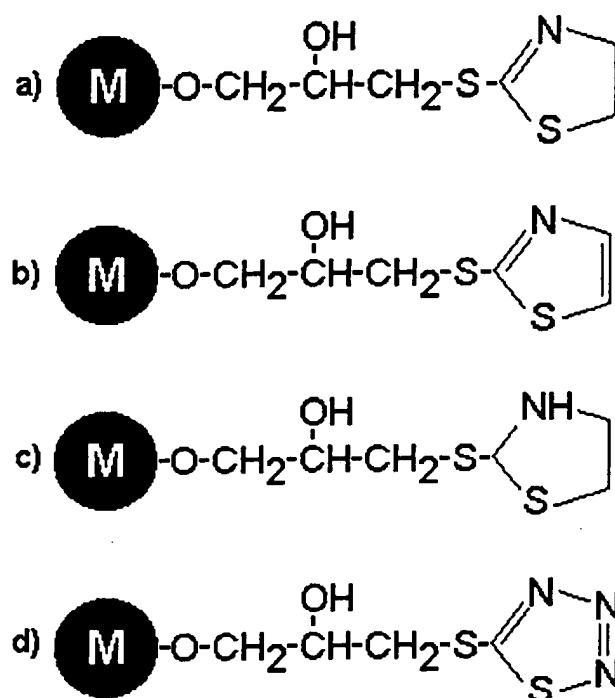


Fig. 6. Representation of heterocyclic thiophilic resins, made from heterocycles containing nitrogen and sulfur atoms as part of the ring [55]. All structures are prepared by coupling mercaptans on epoxy-activated matrix. Mercaptans used here are: "a": 2-mercaptothiazoline; "b": 2-mercaptothiazole; "c": 2-mercaptothiazolidine; "d": 2-mercaptothiazatriazole.

achieved with 10 mM HEPES buffer (pH 7.4) or 10 mM Tris-HCl (pH 7.4). Antibody recovery was between 65% and 96% depending on the nature of the ligand, while the purity, measured by polyacrylamide gel electrophoresis, was estimated by the authors to be close to 90%.

All studies on thiophilic adsorption of immunoglobulins involving aromatic or heterocyclic rings make it evident that adsorption is improved when sulfur atoms are present in the spacer arm and/or in the ligand structure. The specificity for IgG is not extremely high, but it can be modulated by different positioning of atoms. All ligand structures reviewed in this section require the presence of lyotropic salts to promote IgG adsorption; this is a major drawback, as described in Section 4 for more classical thiophilic sorbents. Feed stock conditioning and environmental issues related to the elimination of large amounts of buffer-containing sulfates have until now prevented thiophilic adsorption from becoming the dominant method for large-scale immunoglobulin purification even though it is extremely simple.

While it has been demonstrated that ligands based on pyridine structures contribute significantly to the selective adsorption of antibodies, complex structures have also been proposed as effective sorbents [56–58]. Derivatives of aza-arenophilic gels have been

prepared with dichloro-substituents and hydroxyl groups, and used for antibody purification. As in the case of the previously described thiophilic structures, adsorption happens in the presence of highly concentrated salts. Here, the salt, sodium chloride, is not used to promote the adsorption of antibodies as lyotropic products, but rather to prevent the adsorption of albumin and of other proteins in the sample. Adsorbed antibodies can be eluted with a dilute solution of sodium acetate (pH 4.2). Elution of antibodies can alternatively be achieved in neutral medium with the use of electron-rich compounds, such as triethylamine, mercapto-glycerol, and acetonitrile.

Generally, sulfur atoms are not present on the ligand. Therefore, these structures cannot be called thiophilic, but they are thiophilic when they are end-capped with nucleophiles containing a sulfur atom, such as mercaptans. When mercapto-ethanol is used, the derivative obtained is very effective for adsorbing and separating antibodies [59]. In this case, the use of highly concentrated salt conditions can be avoided during binding and possibly replaced by an intermediate wash to eliminate non-specifically adsorbed impurities.

More recently, other heterocyclic compounds have been described as ligands for the adsorption and purification of antibodies. Divinylsulfone has been used as activating agent containing a sulfur atom, and 3-(2-mercaptoethyl)quinazoline-2,4(1*H*,3*H*)dione was immobilized [60]. This derivative allowed the adsorption of antibodies without addition of lyotropic salts. Desorption was achieved by increasing the pH, contrary to all previous processes. Examples of the purification of immunoglobulins from different species with this material have been described. Scholz et al. [61] have reported another series of possible thiophilic ligands, attached to a matrix preactivated with divinylsulfone. The entire ligand structure contained two sulfur atoms: the first was introduced by the divinylsulfone activation and the second by the heterocyclic compound. Among them were 2-mercapto-pyridine, 2-mercapto-pyrimidine and 2-mercapto-nicotinic acid. Results with mercapto-pyridine differed from those with mercapto-pyrimidine in a higher recovery from the former. To demonstrate the importance of the spacer arm of divinylsulfone, the latter heterocycle was also immobilized after activation with epichlorohydrine. Mercapto-nicotinic acid, attached on a divinylsulfone-activated matrix, showed a significantly higher adsorption capacity than the corresponding material made after activation, using epichlorohydrine. This again reflects the importance of the presence of sulfur atoms for interactions with proteins and especially with antibodies, and allowed, according to the authors, adsorbing antibodies in a salt-independent manner and in neutral medium.

Still within the framework of the above-mentioned use of heterocycles for antibody separation, an older ligand deserves to be cited: Remazol GGL, a reactive dye, attached to agarose beads by a sulfur-containing linker. This was used for the purification of IgG from plasma [62]. The ligand comprised a linker with a sulfur group and a head with aromatic and heterocyclic structures, involving two nitrogen atoms. With this sorbent, IgGs were adsorbed in the presence of 20 mM phosphate buffer (pH 7.4) and were desorbed by increasing the ionic strength with 1 M sodium chloride. The binding capacity for immunoglobulins G was 14 mg/ml of resin; recovery was from 40% to 60% depending on the nature of the sample used and reflecting differences in composition. With this ligand, it has been shown that the interaction did not occur with light



chains. Experimental data suggested that interaction occurs in the N-terminal half of the heavy chain of IgG. Among the contaminants adsorbed on this resin was the thyroxin-binding globulin.

This brief survey of heterocyclic thiophilic ligands clearly shows a trend away from the initial thiophilic aliphatic adsorbents. Linear thiophilic ligands progressively evolved towards the aromatic rings and preferably heterocyclic structures. In parallel, solutions have been sought to adsorb antibodies in physiological conditions rather than using lyotropic salts. Also, binding capacities for antibodies have increased from a few mg to a few dozen of mg per ml of sorbent.

## 7. Hydrophobic charge-induction chromatography

### 7.1. General considerations

Efficient direct capture from crude feed stocks is a critical point for process-scale purification of antibodies, but it is clear that often ionic strength, pH, or antibody concentration of feed stocks are not compatible with currently available sorbents: ion-exchange chromatography requires either dilution to lower the ionic strength or pH adjustment or both [63], although specific situations have been described where only the pH needs to be optimized [64]. Hydrophobic-interaction chromatography requires the addition of lyotropic salts and pseudo-affinity adsorption [65] on immobilized histidine requires either dilution of the crude feed stock or adjustment of pH or both. Only chromatography involving bioaffinity ligands, such as Protein A, allows the direct adsorption of antibodies at near neutrality and physiological ionic strength. However, it should be kept in mind that even with Protein A resins, the separation of certain antibodies requires the addition of sodium chloride to the feed stock along with the adjustment of pH to about 9. As indicated in the previous sections, thiophilic chromatography, in its many variants, provides the basis of using feed stock directly, even though most of the documented data indicate the addition of lyotropic salts. Comparisons with Protein A resins have been documented, but they are of little significance since most of thiophilic sorbents lack the selectivity of Protein A.

What are the chemical modifications of thiophilic resins that will allow approaching the ideal conditions of direct column loading? One of the most recent developments in thiophilic-related chromatography for the separation of antibodies is the so-called hydrophobic charge-induction chromatography, which has come a long way towards achieving this goal [66]. This technique involves a ligand, which combines the thiophilic effect, hydrophobicity, and an ionizable pyridine ring. To understand the benefits of hydrophobic charge-induction chromatography, one must examine the mechanism that underlies this technique.

Burton and Harding [67,68] described the technique as based on adsorption of proteins resulting from a hydrophobic association and desorption resulting from ionic repulsion between the ligand and the adsorbed protein when the pH is changed in an appropriate direction.

A large number of ligands with common hydrophobic properties and ionizable functions were described by the authors. While adsorption is always performed under similar conditions, elution is achieved by switching the pH to higher or lower values, depending on the nature of the ligand and the isoelectric point of the protein. When these ligands contain sulfur atoms, they have properties related to thiophilic chromatography and are thus a further development of this technology.

Features related to the specificity of hydrophobic charge-induction chromatography for antibodies are due to a relatively complex balance between the structure of the ionizable ligand head, the positioning of the sulfur atom, and the structure of the hydrophobicity-inducing moiety.

As reported [66,69,70], ionic strength and pH (if above 6) do not affect adsorption of antibodies. Binding capacity for IgG is best at a pH range between 7 and 9 and at physiological ionic strength; with pure human IgG, it reaches values of 30–40 mg/ml of wet resin at 10% breakthrough. The ligand is attached to the solid matrix via a thioether bond (see below). Specific studies on ligand leakage were conducted with immunoglobulins G under normal conditions of use. It was found that the level of ligand release during elution at pH 4 was below 500  $\mu\text{g/l}$  or below the sensitivity of the analytical HPLC method used.

## 7.2. Mechanism of action

One of the commercially available sorbents for hydrophobic charge-induction chromatography is named MEP HyperCel. The sorbent carries a ligand where the head is a pyridine ring, associated with a sulfur atom separated from the ring by two carbons. This head is attached to a solid matrix by means of a hydrophobic chain. Under physiological conditions of ionic strength and pH, antibodies interact with this immobilized ligand in quite a specific manner with a binding capacity in excess of 30 mg/ml of resin.

The ligand has a  $pK_b$  close to 4.8; when the pH of the environment is below 5, the antibody association strength decreases, and when the pH is decreased below 4.5, the antibody dissociates and is collected. Typically, elution is achieved at a pH close to 4.

The interaction is pH-dependent as indicated in Fig. 7; optimal adsorption occurs when the pH of the solution is between 7 and 8.5, and this is independent of the ionic strength. Adsorption is not only due to the structure of the ligand head (pyridine ring), which is known for its specificity for antibodies (see Section 6), but also by a mild hydrophobic association. The presence of ethylene glycol, in fact, reduces the interaction strength and, at a concentration above 20–30% in buffer, it prevents the adsorption of antibodies (Fig. 8). This behavior is consistent with hydrophobic-based interactions, as described earlier [71]. It has been shown that when a sample containing serum proteins is loaded on the column, albumin is also adsorbed, and it is partially desorbed when the column is washed with a solution of 25 mM sodium caprylate in neutral buffer [66]. Octanoic acid is known to be a competitive hydrophobic agent, which weakens this kind of association. However, it does not prompt desorption of significant quantities of immunoglobulin. Another indication of the presence of hydrophobic interactions is that albumin is adsorbed on hydrophobic solid phases but, as described earlier, it is not adsorbed on pyridine-based thiophilic ligands [33].

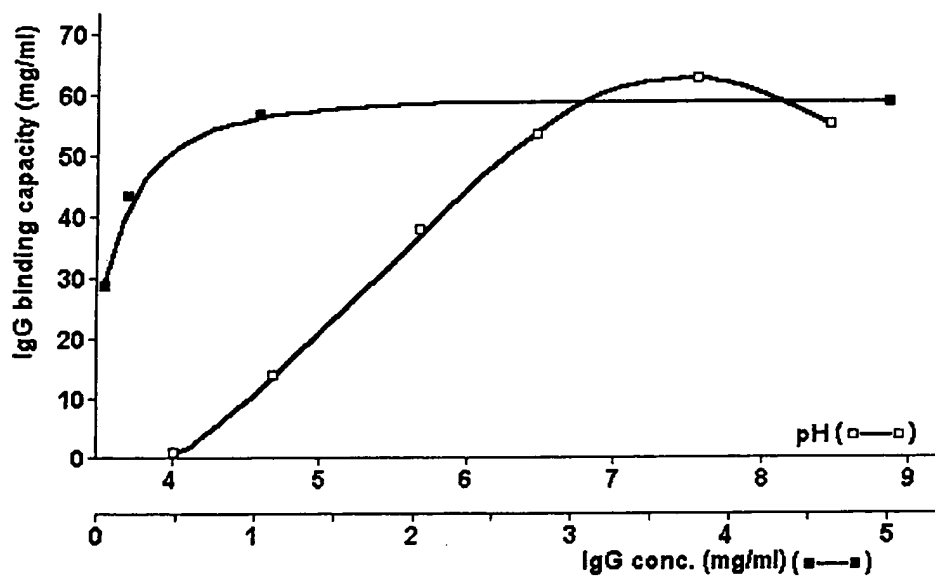


Fig. 7. Dependence of immunoglobulin G binding as a function of pH ( $\square$ — $\square$ ) and of IgG concentration ( $\blacksquare$ — $\blacksquare$ ) for a hydrophobic charge-induction chromatography sorbent (MEP HyperCel). The binding capacity was measured by frontal analysis, using a solution of human immunoglobulins G at a concentration of 5 mg/ml. Buffer system: 10 mM phosphate–citrate of different pH values. Column: 6.6 mm I.D.  $\times$  50 mm. Linear flow rate was 70 cm/h. Calculations were made at 50% breakthrough.

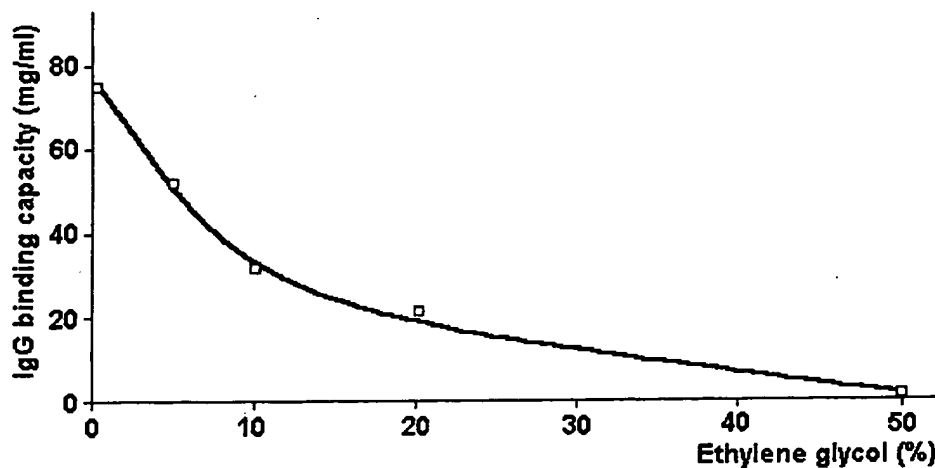


Fig. 8. Binding capacity of human immunoglobulin G on an hydrophobic charge-induction chromatography sorbent (MEP HyperCel) as a function of ethylene glycol concentration. Buffer used: 50 mM Tris–HCl (pH 8.0); IgG concentration: 5 mg/ml in the adsorption buffer added with various amount of ethylene glycol; column: 6.6 mm I.D.  $\times$  50 mm; linear flow rate: 70 cm/h. All calculations were made at 50% breakthrough.

Adsorption in regular buffers without addition of lyotropic salts is achieved because of the association of some ligand features and of the density of the ligand attached to the matrix. The apparent dissociation constant under physiological conditions is close to  $2.9 \times 10^{-7}$  mol/l [70]; this value is similar to that for classical affinity resins, such as Protein A resins [72]. The value is comparable to several affinity constants found for the best thiophilic sorbents when they are used in the presence of lyotropic salts (see Table 3). It should be noted that the density of the pyridine-based ligand on the sorbent was about 100  $\mu$ mol/ml of packed resin, and  $Q_{\max}$  was 48.2 mg of antibody/ml of resin. In this situation and assuming no steric hindrance is present, it is likely that several ligands interact with a single molecule of antibody. Thus, it appears that about 1  $\mu$ mol of ligand is necessary for the adsorption of 2–3 nmol of antibody.

In order to elucidate the interaction area on the antibody molecule, experiments have been performed with antibody fragments. It has been reported [70] that Fab fragments are not adsorbed on MEP HyperCel under given pH and ionic strength conditions (e.g., 100 mM Tris–acetate, pH 5.5), while Fc fragment is adsorbed under the same conditions as whole antibodies and is eluted also under similar conditions. This behavior suggests that there are possible interacting areas in the Fc region having specific structural features that fit into the ligand configuration. Studies are continuing to provide a better understanding whether variants of the ligand induce modification of antibody interaction and thus what is contributed by each single parameter responsible for the antibody adsorption on the solid phase.

### 7.3. Separation of antibodies

A number of examples have been given for the separation of antibodies by hydrophobic charge-induction chromatography. Typically, adsorption from cell culture supernatants occurs without any pH or ionic strength adjustment; the column is then washed with a 50 mM Tris buffer (pH 8). Alternatively, washing can be effected with phosphate buffered saline solutions at pH 7.2 or other buffers of pH > 5–6, containing 0.5 M sodium chloride. An antagonistic effect has been found between pH and ionic strength (Fig. 9). When the pH of the buffer is reduced, the ionic strength must be increased to assure that antibodies remain tightly adsorbed on the resin. This feature can be used for specific washing with the aim of eliminating impurities adsorbed on the column. For instance, if a wash with a mild acid is required to eliminate certain impurities, the presence of a relatively high ionic strength is necessary to prevent the desorption of antibodies. Antibodies are always desorbed by lowering the pH below 4.5 and at relatively low ionic strengths, such as 20–50 mM acetate buffers (pH 4–4.5) or 15–25 mM citrate buffers (pH 3–4.5). In case large amounts of albumin are present in the feed stock, the column is washed after the adsorption phase with a low-ionic-strength buffer at pH 8–8.5, such as 10 mM Tris–HCl (pH 8.5) to weaken the hydrophobic interaction responsible for albumin adsorption.

These conditions can be varied during intermediate washings to desorb undesired impurities; for instance, traces of albumin adsorbed on the column that would otherwise be eluted with the antibodies can be washed out with diluted solutions of caprylic acid

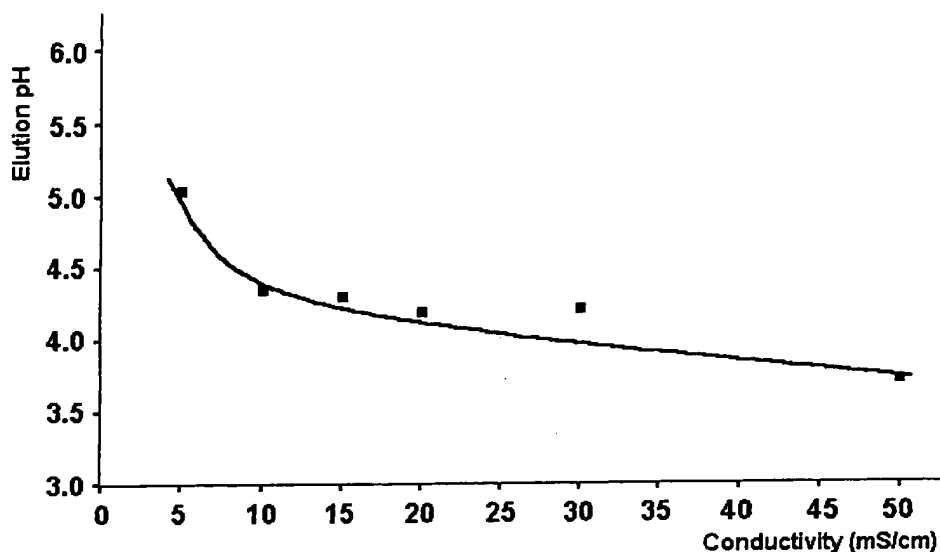


Fig. 9. Elution pH for human polyclonal IgG as a function of ionic strength on a column of MEP HyperCel. Separations were performed using a linear elution gradient of pH, made from two buffers: 10 mM phosphate (pH 8.5) and 10 mM citrate (pH 3.5). The ionic strength was modulated by adding various amounts of sodium chloride to the buffer system. Separations were carried out at different ionic strengths, and the elution pH was determined at 30% of first half of IgG peak. Column: 6.6 mm I.D.  $\times$  50 mm; linear flow rate: 70 cm/h.

(e.g., 25 mM) at pH 7. Elution can be modulated by using different buffers at low pH (e.g., acetate, citrate, glycine); as a general rule, the higher the ionic strength of the buffer, the lower the pH of the buffer must be to achieve complete elution of antibodies.

Antibodies produced in ascites are separated at levels of purity up to 80–85% [66]. Monoclonal antibodies were separated from transgenic goat milk in high purity and yield [69]. Polyclonal antibodies were also separated from sweet whey and serum (results not shown) in a purity ranging from 60% to 65%. In these cases, the reduction of adsorbed impurities may require specific intermediate washings. Several antibodies have been isolated from cell culture supernatants in a single step at a purity of over 95% when cells were cultured in protein-free media, and at a purity of ca. 70% when the culture medium contained fetal bovine serum [66].

An example of antibody separation is illustrated in Fig. 10. In this case, the load was a cell culture supernatant containing fetal bovine serum at a concentration of 5%. The antibody concentration in the feed stock was 45  $\mu$ g/ml. Under the conditions described in the figure legend, the antibody was well captured while most of protein impurities were found in the flow through and, after elution, the purity of collected IgG was estimated close to 80–85%. Due to the very low level of expression, the column load was of 360 ml of feed stock/ml of resin. Intermediate washes with both distilled water and with 25 mM sodium caprylate were used to weaken hydrophobic interaction with other components of the cell culture medium, particularly albumin. This approach contributed significantly to the final purity of the separated antibody.

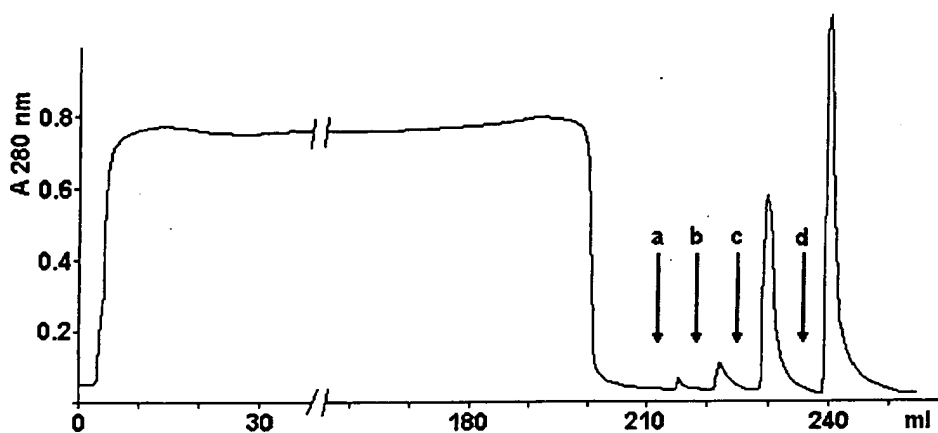


Fig. 10. Purification of an IgG1 monoclonal antibody from a cell culture supernatant, containing 5% fetal bovine serum, on a column of MEP HyperCel. Cell culture supernatant (250 ml) was directly injected into a column (3.0 mm I.D.  $\times$  10 mm) without prior treatment; this corresponded to 375 column volumes. The concentration of IgG1 was 0.045 mg/ml. The column was first washed with a 50 mM Tris-HCl buffer (pH 8.0) (arrow "a") followed by two other washings: (a) 0.25 M sodium caprylate in Tris-HCl buffer, and (b) distilled water (see arrows "b" and "c", respectively). Elution of antibodies (arrow "d") was then obtained using a 50 mM acetate buffer (pH 4.0). The column was finally cleaned with 1 M solution of sodium hydroxide. Linear flow rate was 70 cm/h. The purity of eluted IgG1 was estimated to be close to 84% (SDS-PAGE and size exclusion-HPLC).

#### 7.4. Comparison with Protein A resin

Although both MEP and Protein A ligands are used for antibody separation, their interaction mechanism with IgG antibodies is different. With the former ligand, the interaction likely involves the pyridine ring. Moreover, the presence of a sulfur atom and the hydrophobicity of the spacer contribute for the interaction with antibodies. Conversely, Protein A interaction involves a sequence of 32 amino acids, most of them predominantly hydrophobic. Phe, Tyr, Leu and Ile are representative of the binding site, while few other amino acids, (Gln, Lys, Arg and Asn) contribute for either hydrogen bonds or ion pair [73]. From these fundamental differences, hydrophobic charge-induction chromatography with the specific sorbents for antibody MEP HyperCel is not claimed to be accomplished with a biomimetic ligand related to *S. aureus* Protein A. Comparative studies to check the difference in the purification of antibodies have been done side-by-side.

Fig. 11 represents a comparative experiment for the capture separation of monoclonal IgG1 from cells cultured in a protein-free medium. The adsorption buffer for Protein A contained a large amount of sodium chloride and glycine, and the pH was raised to 8.9 to promote the adsorption of antibodies, while in the case of MEP HyperCel, the feed stock was loaded without adjustment of pH and without addition of glycine or salt. In both cases, elution was performed by lowering the pH, using 100 mM acetic acid (pH  $\sim$  3) and 50 mM acetate buffer (pH 4.0), respectively, for Protein A resin and for the hydrophobic charge-induction chromatography resin. The difference in pH values for

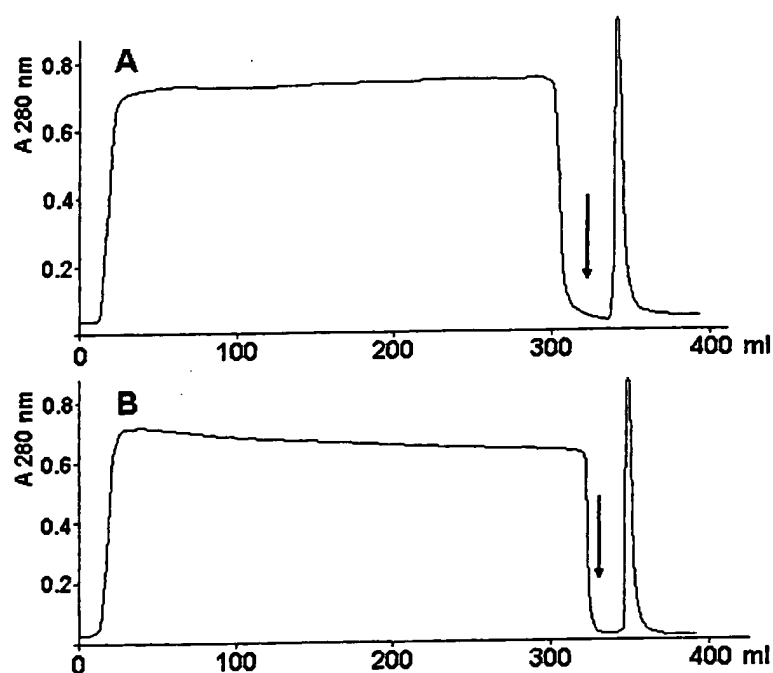


Fig. 11. Comparative separation of monoclonal IgG1 antibodies from a protein-free cell culture supernatant on MEP HyperCel (A) and Protein A Ceramic HyperD (B). A: 300 ml of cell culture supernatant, containing 200  $\mu\text{g/ml}$  IgG1, was injected without preliminary pretreatment in a column of MEP HyperCel at a linear flow rate of 60 cm/h. The column was then washed with a phosphate buffer saline at pH 7 and antibodies desorbed with a 50 mM acetate buffer pH 4 (arrow). B: 300 ml of cell culture supernatant, containing 200  $\mu\text{g/ml}$  IgG1, was first added with glycine up to 1 M concentration and with sodium chloride up to 2 M concentration, and the pH was adjusted to 8.9. This preconditioned feed stock was injected into a column of Protein A Ceramic HyperD at a linear flow rate of 60 cm/h. The column was then washed with phosphate-buffered saline at pH 7 and antibodies were desorbed with 100 mM acetic acid solution (arrow). Comparative purity and recovery are described in Table 4.

desorption, though not very different, is of particular relevance since it prevents the formation of antibody aggregates or partial denaturation and allows loading the eluted peak directly onto a cation exchanger for further polishing without prior modification of pH. Table 4 summarizes the experimental results. It should be noted that the binding capacity for this particular antibody was 3–4 times lower with Protein A resin (for polyclonal antibodies, the binding capacity for both resins was similar). Purity was also similar, as attested by analytical HPLC and SDS polyacrylamide gel electrophoresis.

Other results obtained for cell culture supernatants containing fetal bovine serum showed that the purity of antibodies was greater with the use of Protein A resin (95% purity with Protein A resin against 80–85% purity for the hydrophobic charge-induction chromatography resin). However, antibodies separated on hydrophobic charge-induction resin contained lower amounts of large-molecular-weight aggregates than those from Protein A resin, as attested by size exclusion-HPLC (ca. 4.5% against ca. 8%).

Table 4

Comparative data between MEP HyperCel sorbent and Protein A Ceramic HyperD for the separation of monoclonal IgG1 from protein-free cell culture supernatant

	Binding capacity (mg IgG1/ml)	Loading volume (ml sample)	Elution pH	Elution volume (column volume)	Purity (%)	Recovery (%)
Protein A Ceramic HyperD <sup>a</sup>	10	150	3.0	1.8–2	> 98	86
MEP HyperCel <sup>b</sup>	38	150	4.0	1.8–2	> 98	96

<sup>a</sup> Binding buffer: M glycine–NaOH, 2 M sodium chloride, pH 8.9.

<sup>b</sup> Binding buffer: 50 mM Tris–HCl, pH 8.0.

A comparison between hydrophobic charge-induction and Protein A resin chromatography was recently published [69]. It showed that when the feed stock was the ultrafiltrate of a transgenic milk, antibodies from Protein A column were not significantly better purified, as tested by regular and SDS-reduced polyacrylamide gel electrophoresis and by Western blot analysis. The purity of antibodies was estimated to be  $\geq 95\%$ . DNA clearance was also reported to be very effective [69] due in part to the fundamental mechanism of the hydrophobic charge-induction chromatography ligand. At the loading pH, conditions for DNA adsorption on the resin are not satisfied, and most of the nucleic acid molecules are found in the flow-through. During elution at pH 4, the sorbent carries a predominantly positive charge, and any traces of nucleic acid still present would be expected to bind to the column while antibodies are desorbed. Remaining traces of still tightly adsorbed DNA would then be expected to be desorbed during the strong NaOH wash used at the conclusion of the separation cycle to clean the column.

Due to the greater chemical stability and binding capacity associated with the direct loading without prior sample treatment, the hydrophobic charge-induction resin had, in this case, decisive advantages over the Protein A resin. Nevertheless, even when adsorption conditions are similar (physiological conditions for both columns), additional advantages differentiate MEP HyperCel resin from Protein A resin, e.g., leakage phenomena related to either possible enzymatic breakdown of Protein A or to detachment from the resin as a result of link hydrolysis, or both, that contaminate separated antibodies. Additionally, Protein A resin is notorious for high price, difficulties in sanitization, and limited number of separation cycles.

#### 7.5. Future of hydrophobic charge-induction chromatography technology

Hydrophobic charge-induction chromatography technology, as it is described above, comes in a large number of variations that can be designed to improve its specificity for antibodies and related proteins. If the mechanism of action is confirmed as an association of the whole ligand with the Fc moiety of antibodies, it could be envisioned that fusion proteins with Fc might also be purified at similar performance levels.

As for ligand design, the hydrophobic character could be increased, or the sulfur positioning changed, or even the pyridine ring replaced by other, more sophisticated



heterocyclic structures that would increase the specificity for antibodies of different classes. In the case of antibodies that are sensitive to acid elution, new ligands, based on the same mechanism, could be designed in such a way that the elution would be performed by increasing the pH instead. This approach could be beneficial any time impurities in the feed stock interfere with the antibody adsorption or when specific impurities are also adsorbed on the resin, depending on the nature of the feed stock and the expression system used for the production of monoclonal antibodies.

## 8. Conclusion and prospects

Over the years, thiophilic chromatography for the purification of antibodies has evolved in such a manner that applications to large-scale separations are now possible under good handling and economical conditions. The purity of antibodies is satisfactory especially for selected feed stocks or when thiophilic chromatography is combined with other, complementary separation methods.

Medical applications of antibodies frequently involve treatment with large doses for relatively long periods of time. This situation influences not only the choice of the method of expression, but also the approaches to purification. Large amounts of antibodies call for large columns with high-binding-capacity sorbents. Specificity of the sorbent and its use at the capturing phase under physiological conditions are the major issues in this domain. The treatment of large volumes of feed stocks for short periods of time means availability of non-compressible sorbents with high binding capacity to limit the column size and minimize the operation time. It is envisioned that thiophilic-related technologies will be used, not only in packed-bed, but also in fluidized-bed modes to eliminate the clarification of feed stocks prior to chromatographic fractionation. Hence, in the coming years, new thiophilic ligands will probably be attached to denser solid phases, suitable for fluidized-bed operation.

Antibodies are relatively complex molecular structures, containing several regions that can be easily modified; this has been demonstrated by the changes from rodent to humanized forms which form the basis of the present rapid developments of antibodies as therapeutics. Protein engineering allows changes in the antibody molecules, producing new generations of fusion constructs better targeted to given diseases. Immunotoxins (antibodies carrying toxic drugs or biodrugs), and abzymes (antibodies carrying enzymatic activities) are specific examples of proteins that induce changes in expression systems and downstream processing. Antibodies themselves are currently modified into chimeric antibodies, heterodimers; antibody fragments may be expressed directly as such rather than obtained through proteolytic breakdown of native antibodies. Since feed streams, produced by recombinant technologies, will probably remain dilute for a long time, there is a need for ligand structures, which provide high affinity constants without the addition of lyotropic salt. Moreover, evolution of expression technology (transgenic animals, transgenic plants, etc.) will additionally pose new challenges to the downstream processing of recombinant antibodies or antibody-derived molecules. Thiophilic chromatography and all its possible variants should play an important role in downstream processing of these molecules. It is envisioned that continued research on the design of

new thiophilic ligands will be necessary. Present-day combinatorial chemistry might provide an avenue for research: combinatorial ligand structures might be made around scaffolds that have in common one or more sulfur groups, associated with nitrogen-containing organic structures. Moreover, the recent, important developments of hydrophobic charge-induction chromatography, described in Section 7, will probably contribute to new and better purification procedures.

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